MiR-25 exerts cardioprotective effect in a rat model of myocardial ischemia-reperfusion injury by targeting high-mobility group box 1

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Abstract

Background: We previously confirmed the targeting of high-mobility group box 1 (HMGB1) by miR-25. This project aims to further investigate whether miR-25 improves myocardial ischemia-reperfusion injury (IRI) in vivo by targeting HMGB1.

Methods: A rat model of myocardial IRI was established by the ligation of the left anterior descending coronary artery for 45 minutes followed by 2, 4, or 6 hours reperfusion. The expression of miR-25, HMGB1, and apoptosis-related proteins in the myocardium was determined by quantitative real-time polymerase chain reaction (PCR) and western blotting. The activities of myocardial enzymes and the release of inflammatory cytokines were evaluated by enzyme-linked immunosorbent assay. Evans blue/triphenyltetrazolium chloride double staining was performed to assess infarct size. Myocardial apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling staining.

Results: MiR-25 expression was significantly downregulated, while HMGB1 was highly expressed at the mRNA and protein levels in myocardial tissues after induction of the IRI model. MiR-25 agomir administration suppressed the expression of HMGB1 in myocardial tissues. Furthermore, administration of both miR-25 agomir and lentivirus-mediated short hairpin RNA (shRNA) interference targeting HMGB1 sh-HMGB1 resulted in reduced serum myocardial enzyme activities, cytokine secretion, and myocardial apoptosis during myocardial IRI.

Conclusion: MiR-25 mitigated myocardial IRI-induced damage by targeting HMGB1.

Keywords: Animal experiment; Ischemia-reperfusion injury; Myocardium

1. INTRODUCTION

Coronary heart disease is an extremely serious ischemic heart disease caused by insufficient blood supply to cardiac tissues and remains the leading cause of death and disability worldwide.[1,2] Following coronary artery obstructions, an ischemic environment gradually forms in surrounding cardiac tissues, which can trigger cardiac apoptosis, leading to the loss of functional cardiomyocytes.[3] It is important to note that the loss of cardiac myocytes directly contributes to cardiac remodeling, which can be aggravated by the irreversible damage to myocardial cells, resulting in heart failure. It has become increasingly clear that the prevention of myocardial ischemia-reperfusion injury (IRI) is essential for the treatment of ischemic heart diseases.[4] However, the molecular mechanisms underlying myocardial IRI are far from understood. To date, emerging efforts have been made to identify crucial factors that are involved in the regulation of myocardial apoptosis following IRI, and pharmacological therapies targeting some regulators have been developed for clinical trials to prevent cardiac dysfunction.[5,6] High-mobility group box 1 (HMGB1) emerged as a proinflammatory cytokine named for its high migration speed in polyacrylamide gel electrophoresis. In recent years, abundant studies have provided strong evidence that HMGB1 participates in cell replication, differentiation and maturation, DNA repair and recombination, steroid hormone regulation, and gene transcription regulation.[7,8] During ischemia, HMGB1 is released by inflammatory cells and necrotic cardiomyocytes, which initiates proinflammatory signaling pathways.[9,10]

MicroRNAs are small noncoding RNAs of 21 to 23 nucleotides that are involved in posttranscriptional regulation of eukaryotic gene expression by binding to the 3′-untranslated region (UTR) of the mRNAs of their target genes.[11] A growing body of evidence suggests that the aberrant expression of the miR-106b-25 family, located in a 515 bp region on chromosome 7q22, indicates its potential role in myocardial IRI.[12] We previously confirmed the cardioprotective mechanism of miR-25 via an HMGB1-mediated effect on the transforming growth factor-β1/Smad3 signaling pathway.[13] In addition, Qin et al recently reported that the inhibitory effect of miR-25 downregulation on cardiomyocyte proliferation and migration was due to the direct targeting of Bim.[14] However, the regulatory effect of miR-25 on the HMGB1 protein in damaged heart tissues following myocardial IRI remains unclear. Herein, we mainly explored the cardioprotective mechanism of the miR-25/HMGB1 axis in myocardial IRI rats.

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2. METHODS

2.1. Rat model of myocardial IRI

Male Sprague-Dawley rats (weighing 250–300 g) fed standard rodent chow under specific pathogen-free conditions were used in our study. The rats were obtained from the Laboratory Animal Center of Guizhou Provincial People's Hospital. A rat model of myocardial IRI was established. Briefly, all rats were anesthetized with sodium pentobarbital (40 mg/kg) by intraperitoneal injection and underwent a standard midline thoracic incision between the third and fourth ribs. A 6-0 silk suture was placed around the root of the left anterior descending coronary artery and was ligated for 45 minutes to induce ischemia, while the sham-operated rats underwent an identical procedure except for the suture ligation. Rats were sacrificed following reperfusion for 2, 4, and 6 hours (n = 6 for each group). Cardiac tissues were harvested for determination of miR-25 and HMGB1 expression using quantitative real-time PCR and western blot. The experimental procedures were approved by the Animal Care and Use Committee of Guizhou Provincial People's Hospital.

2.2. Administration of miR-25 agomir and lentivirus-mediated shRNA interference targeting HMGB1

To identify the role of miR-25 in IRI rats, miR-25 agomir was used to upregulate endogenous miR-25 expression in vivo. To identify the role of HMGB1 in IRI rats, lentivirus-mediated shRNA interference targeting HMGB1 (LV-sh HMGB1) was used to downregulate endogenous HMGB1 expression in vivo. Sprague-Dawley rats received miRNA agomir negative control or miR-25 agomir (both from RiboBio Co., Guangzhou, China) at a single dose of 10 mg/kg body weight, or LV-sh negative control, and LV-sh HMGB1 through tail vein injection at 48 hours before regional ischemia (n = 6 for each group). After 48 hours, IRI was induced in these rats, and blood and heart tissues were isolated for experimental measurements.

2.3. RNA isolation and quantitative real-time PCR

Total RNA was extracted from hearts using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into complementary DNA using a reverse transcription kit (Takara, Beijing, China) according to the manufacturer's protocol. The relative quantification of miR-25 and HMGB1 expression levels was determined using an ABI 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) and calculated by the $2^{-\Delta\Delta Ct}$ method. U6 and β-actin served as internal controls, respectively. The primer sequences were as follows: miR-25, forward 5′-GTGTTGAGAGGGCGGAGACTT-3′ and reverse 5′-TCAGACCGAGACAAGTGCAA-3′; U6, forward 5′-GCTTCGGCAGCACATATACTAAAAT-3′ and reverse 5′-CGCTTCACGAATTTGCGTGTCAT-3′; HMGB1, forward 5′-GGAGAGTAATGTTACAGAGCGG-3′ and reverse 5′-AGGATCTCCTTTGCCCATGT-3′; and β-actin, forward 5′-TGTGATGGTGGGAATGGGTCAGAA-3′ and reverse 5′-TGTGGTGCCAGATCTTCTCCATGT-3′.

2.4. Western blot

Total protein was separated from myocardial tissues using a Radio-Immunoprecipitation Assay (Santa Cruz Biotechnology, Santa Cruz, CA, USA) following centrifugation. Total protein extract was incubated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking with 5% skim milk in Tris-buffered saline with Tween-20 at room temperature for 1 hour, the membranes were probed with primary antibodies against HMGB1 (#6893), caspase-3 (#9662) and B-cell lymphoma-2 (Bcl-2) (#15071) (1:1000 dilution; Cell Signaling Technology, Boston, MA, USA) at 4°C overnight and then incubated with horseradish peroxidase–conjugated secondary antibodies at room temperature for 2 hours. Band intensities were standardized against β-actin, and the relative density was analyzed on a Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, CA, USA) using enhanced chemiluminescence reagent (Thermo Scientific, Shanghai, China).

2.5. Enzyme-linked immunosorbent assay

Blood samples were centrifuged at 3000 rpm for 10 minutes at 4°C to harvest serum. The activities of creatine kinase muscle-brain fraction (CK-MB) and lactic acid dehydrogenase (LDH) in serum were detected by commercial kits (Shanghai Enzyme Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The results were expressed as U/mL or U/L. The production of the proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β was measured using quantitative ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Each blood sample was tested in duplicate.

2.6. Assessment of myocardial infarct size

The myocardial infarct size was evaluated by Evans blue/triphenyltetrazolium chloride (TTC) double staining (Sigma-Aldrich, St. Louis, MO, USA) as described previously.[15] Briefly, after 2 hours of reperfusion, the left anterior descending coronary artery was completely ligated, and 2.0% Evans blue dye was injected via the jugular vein. After 5 minutes of staining, the hearts were taken immediately and stored at –80°C for 20 minutes and then sliced into 2-mm sections and fixed in 4% paraformaldehyde overnight. The slices were soaked in 1% TTC solution for 30 minutes in the dark and fixed in 10% formaldehyde overnight. The nonischemic area (blue), the myocardium area at risk (red), and the infarct area (white) were measured using Image-Pro Plus 6.0 software (Media Cybernetic, Rockville, MD, USA).

2.7. Apoptosis detection

Myocardial apoptosis was quantified by an in situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis detection kit (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China) according to the manufacturer's instructions. Formalin-fixed, paraffin-embedded myocardium sections were prepared for TUNEL assay. The number of TUNEL-positive cardiomyocytes was calculated under an inverted microscope (Nikon Corporation, Tokyo, Japan).

2.8. Statistical analysis

All statistical analyses were conducted using SPSS software version 19.0 (IBM, Chicago, IL, USA). One-way analysis of variance was used to analyze differences among groups. All results were expressed as the mean ± SD, and $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. MiR-25 was downregulated, whereas HMGB1 was upregulated in a rat model of myocardial IRI

To examine whether miR-25 and HMGB1 were involved in myocardial IRI, we first assessed the expression of miR-25 and HMGB1 in a rat myocardial IRI model. After 2, 4, and 6 hours of myocardial reperfusion, the myocardial miR-25 expression was significantly decreased (Fig. 1A), while the mRNA (Fig. 1B) and protein levels (Fig. 1C) of HMGB1 were remarkably upregulated in the IRI group compared with the sham group. Furthermore, the downregulation of miR-25 and upregulation of HMGB1 following IRI were time dependent.

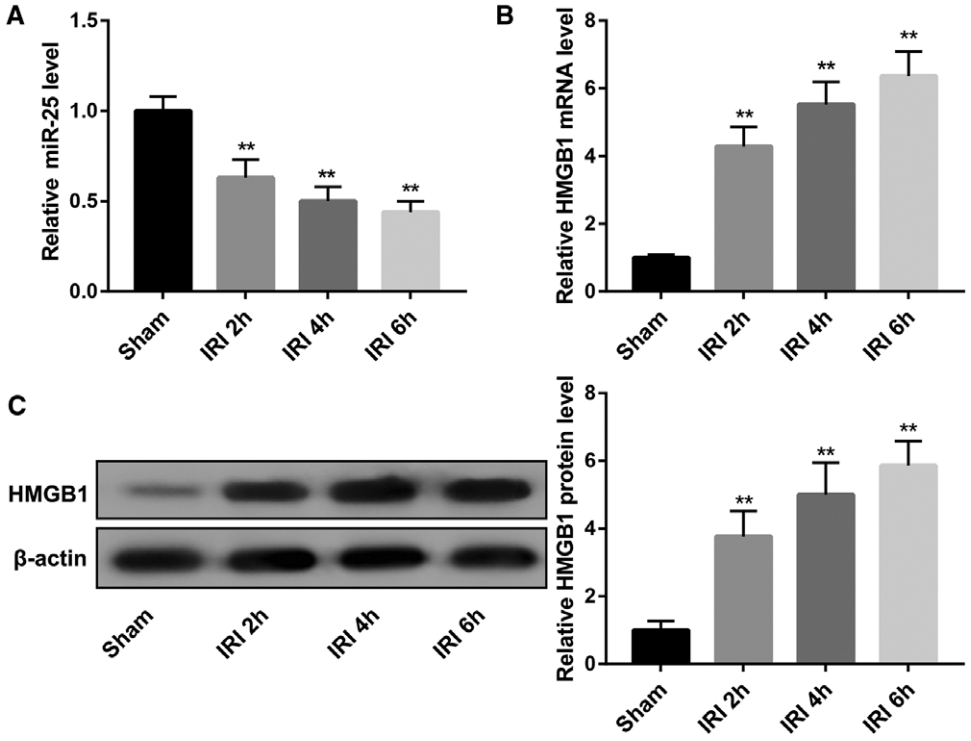

Fig. 1. MiR-25 and high-mobility group box 1 (HMGB1) changed inversely in a rat model of myocardial ischemia-reperfusion injury (IRI). The miR-25 expression (A) and the mRNA and protein levels of HMGB1 (B, C) in myocardial tissues obtained from Sprague-Dawley rats subjected to ischemia for 45 min followed by reperfusion for 2, 4, and 6 h and sham-operated rats using quantitative real-time PCR (qRT-PCR) and western blot. $^{**}p < 0.01$ vs the sham group. n = 6 for each group.

3.2. MiR-25 agomir administration reduced serum myocardial enzyme activities and inflammatory cytokine production during myocardial IRI

Next, we evaluated the potential role of miR-25 in myocardial IRI. To this end, we determined whether restoring miR-25 expression via tail vein injection of miR-25 agomir could exert a protective effect on cardiac function. Compared with the sham group, the IRI group exhibited higher serum levels of cardiac function biomarkers, including CK-MB (Fig. 2A) and LDH (Fig. 2B), which were suppressed by injection of miR-25 agomir. Moreover, treatment with miR-25 agomir abrogated the I/R (2 hours reperfusion)-induced elevation of serum levels of the inflammatory cytokines TNF-α (Fig. 2C) and IL-1β (Fig. 2D). Together, these data indicated that miR-25 agomir reduced serum myocardial enzyme activities and inflammatory cytokine production during myocardial IRI.

3.3. MiR-25 agomir administration alleviated myocardial IRI-induced pathological damage and apoptosis

Given that the upregulation of miR-25 was associated with IRI-induced cardiac injury, we further investigated whether miR-25 agomir administration was capable of attenuating infarct size and apoptosis in myocardial tissues. To address this, myocardial infarct size was evaluated by Evans blue/TTC double staining. As shown in Fig. 3A, no myocardial infarcts were observed in the sham group, while approximately 40% of examined tissues showed myocardial infarct areas in the IRI group. In contrast, the percentage of infarct areas was significantly reduced after the administration of miR-25 agomir. Furthermore, TUNEL staining evaluating myocardial apoptosis revealed that IRI induction markedly increased the number of apoptotic cells, whereas miR-25 agomir administration resulted in a prominent reduction in apoptosis in myocardium tissues (Fig. 3B). Additionally, western blot results suggested that IRI induction significantly elevated the protein level of proapoptotic caspase-3 but reduced that of antiapoptotic Bcl-2. Notably, miR-25 agomir administration effectively reversed the IRI-mediated regulation of caspase-3 and Bcl-2 (Fig. 3C). Collectively, these data suggested that miR-25 agomir administration alleviated myocardial IRI-induced pathological damage and apoptosis. Moreover, the IRI-induced increase in HMGB1 protein levels was notably reduced by miR-25 agomir administration (Fig. 3C), indicating that miR-25 agomir-mediated alleviation of IRI was associated with HMGB1 downregulation.

3.4. LV-sh HMGB1 administration reduced myocardial IRI-induced serum myocardial enzyme activities, inflammatory cytokine production, and myocardial apoptosis

Finally, we determined whether downregulating HMGB1 expression via tail vein injection of LV-sh HMGB1 could exert a protective effect on myocardial IRI. The data revealed that injection of LV-sh HMGB1 suppressed I/R (2 hours reperfusion)-induced elevation of the serum levels of cardiac function biomarkers CK-MB (Fig. 4A) and LDH (Fig. 4B) and inflammatory cytokines TNF-α (Fig. 4C) and IL-1β (Fig. 4D). Furthermore, TUNEL staining showed that LV-sh HMGB1 administration resulted in a prominent reduction in I/R (2 hours reperfusion)-induced apoptosis in myocardium tissues (Fig. 4E). Together, these data indicated that the administration of sh-HMGB1 reduced myocardial IRI-induced serum myocardial enzyme activities and inflammatory cytokine production and alleviated myocardial IRI-induced myocardial apoptosis.

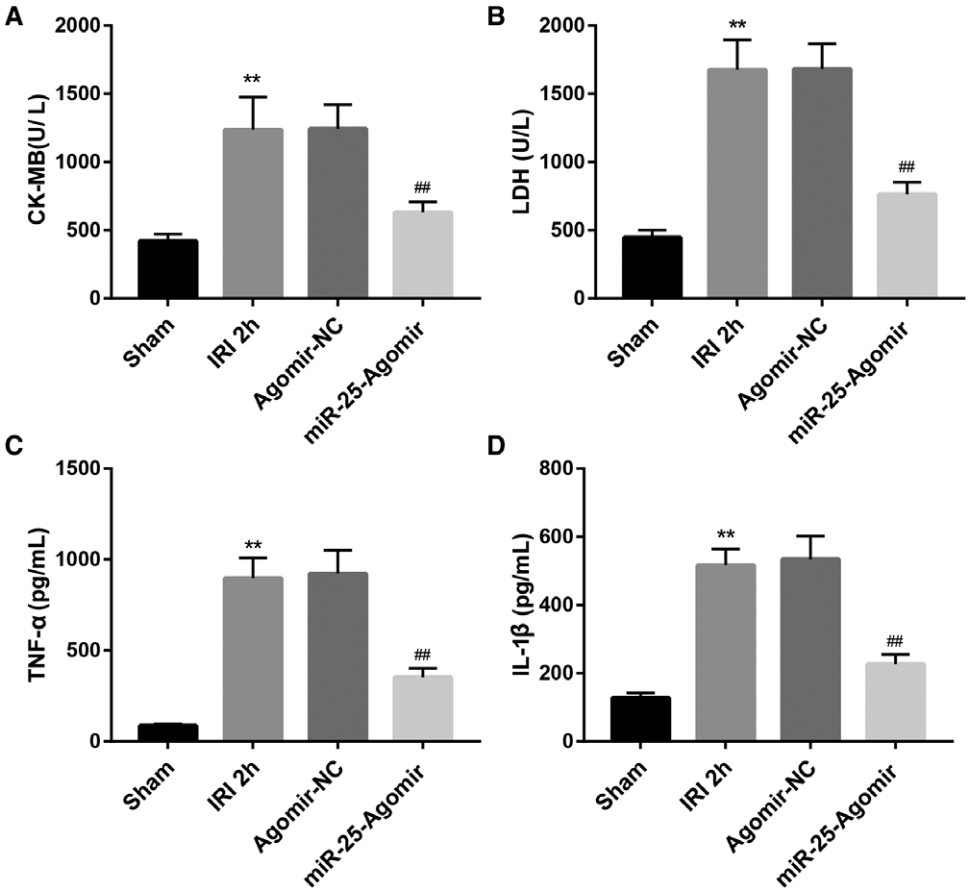

Fig. 2. MiR-25 agomir administration reduced serum myocardial enzyme activities and inflammatory cytokines production during myocardial ischemia-reperfusion injury (IRI). Enzyme-linked immunosorbent assay (ELISA) detection of serum levels of creatine kinase muscle-brain fraction (CK-MB) (A), lactic acid dehydrogenase (LDH) (B), tumor necrosis factor-α (TNF-α) (C), and interleukin (IL)-1β (D) in Sprague-Dawley rats injected of agomir negative control (agomir-NC) or miR-25-Agomir (10 mg/kg) at 48 h before ischemia-reperfusion (I/R; reperfusion for 2 h) induction. $^{**}p < 0.01$ vs the sham group; $^{\#\#}p < 0.01$ vs the Agomir-NC group. n = 6 for each group.

4. DISCUSSION

In the present study, our findings identified the protective effect of miR-25 on I/R-induced myocardial injury, which was indicated by decreased activity of CK-MB and LDH, infarct size, and cardiomyocyte apoptosis. The results also demonstrated that pretreatment with miR-25 agomir could decrease I/R-induced inflammatory cytokine production and downregulate myocardial HMGB1 expression.

Studies have increasingly revealed that miRNAs perform their biological functions by targeting protein-coding mRNAs. Compelling evidence has delineated the potential of miRNAs as therapeutic targets in the treatment of cardiovascular diseases, including atherosclerosis,[16] myocardial infarction,[17] and heart failure.[18] Recently, the concept of using miRNAs in treating myocardial IRI has emerged. For example, downregulation of miR-327 via adenovirus transfection in vivo inhibited the activation of the TLR4-MyD88-NF-κB signaling axis by the targeting of RP105, which subsequently attenuated myocardial IRI-induced myocardial infarct and cardiac inflammation.[19] Wang et al demonstrated that overexpression of miR-135a in myocardial tissues exhibited protective effects on myocardial I/R injury, as evidenced by decreased infarct size and cardiomyocyte apoptosis.[20] Wu et al showed that upregulated miR-202-3p improved the antioxidative ability and alleviated the inflammatory response to suppress myocardial cell apoptosis and myocardial fibrosis by activating the transforming growth factor-β1/Smads signaling pathway, thus ameliorating the myocardial IRI.[21] Here, we showed that miR-25 expression was downregulated in I/R myocardial tissues compared with the tissues of sham-operated rats. This result was consistent with previous reports showing that miR-25 was downregulated in IRI model rats and hypoxia-stimulated cardiomyocytes, and knockdown of miR-25 could reduce the proliferating and migrating ability of cardiomyocytes and promote apoptosis.[14] However, the cardioprotective effect of miR-25 was limited to in vitro validation. A systemic delivery of miR-25 had not been tested. In this study, intravenous pretreatment with miR-25 agomir reduced serum cardiac enzyme (CK-MB and LDH) activities, suggesting that exogenous miR-25 could improve cardiac function. In addition, intravenous injection of miR-25 agomir decreased the serum levels of TNF-α and IL-1β, which are important pathophysiological components of myocardial IRI, suggesting that miR-25 could inhibit myocardial inflammation. Furthermore, exogenous administration of miR-25 agomir before IRI led to a decrease in myocardial infarct size and myocardial cell apoptosis.

We previously identified HMGB1 as a direct target of miR-25.[13] There is emerging evidence that the HMGB1-related pathway plays a pathogenic role in triggering cardiomyocyte apoptosis during myocardial IRI by promoting the release of inflammatory cytokines.[22,23] Therefore, extracellular HMGB1

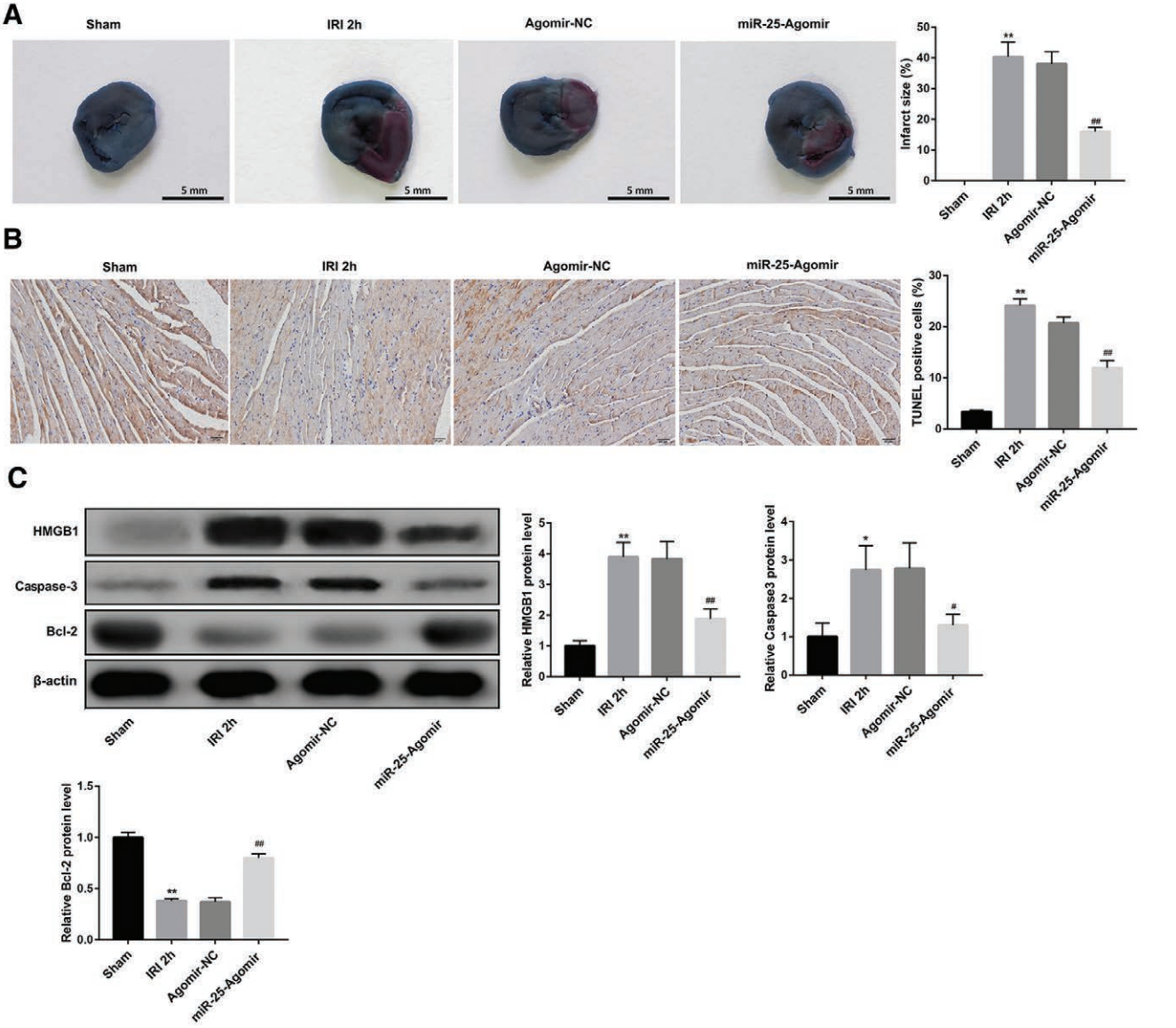

Fig. 3. MiR-25 agomir administration alleviated myocardial ischemia-reperfusion injury (IRI)–induced pathological damage and apoptosis. Representative images of Evans blue/triphenyltetrazolium chloride (TTC) staining (A) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (B) in myocardial tissues and the calculation of infarct size and apoptotic cells. A, The myocardial infarct size was evaluated by Evans blue/TTC double staining. The percentage of infarct areas was significantly reduced after the administration of miR-25 agomir. Scale bar: 5 mm. B, TUNEL staining was performed to evaluate myocardial apoptosis. Scale bar: 20 μm. IRI induction markedly increased the population of apoptotic cells, whereas miR-25 agomir administration resulted in a prominent reduction of apoptosis in myocardium tissues. C, The protein levels of high-mobility group box 1 (HMGB1), caspase-3, and B-cell lymphoma-2 (Bcl-2) in myocardial tissues were determined by western blot. $^{*}p < 0.05$, $^{**}p < 0.01$ vs the sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs the agomir negative control (Agomir-NC) group. n = 6 for each group.

participates in the pathogenesis of inflammation and enhances myocardial IRI.[24] Considering the interaction between miR-25 and HMGB1, the HMGB1 levels in normal and I/R cardiomyocytes were assessed. Our study showed that IRI model rats exhibited higher HMGB1 mRNA and protein levels in the myocardium. This study further validated that the upregulation of HMGB1 in IRI tissues was attenuated by miR-25 agomir. Moreover, LV-sh HMGB1 administration reduced myocardial IRI-induced serum myocardial enzyme activities, inflammatory cytokine production, and myocardial apoptosis. Together, these results support the notion that miR-25 is an important regulator of I/R-induced cardiac injury and that the effect of miR-25 in attenuating IRI might be attributed to targeting HMGB1.

In conclusion, this study demonstrated that miR-25 exerts protective effects on myocardial IRI by targeting HMGB1. Administration with miR-25 agomir in vivo markedly improved cardiac dysfunction and myocardial inflammation and decreased infarct size and cardiomyocyte apoptosis induced by myocardial IRI.

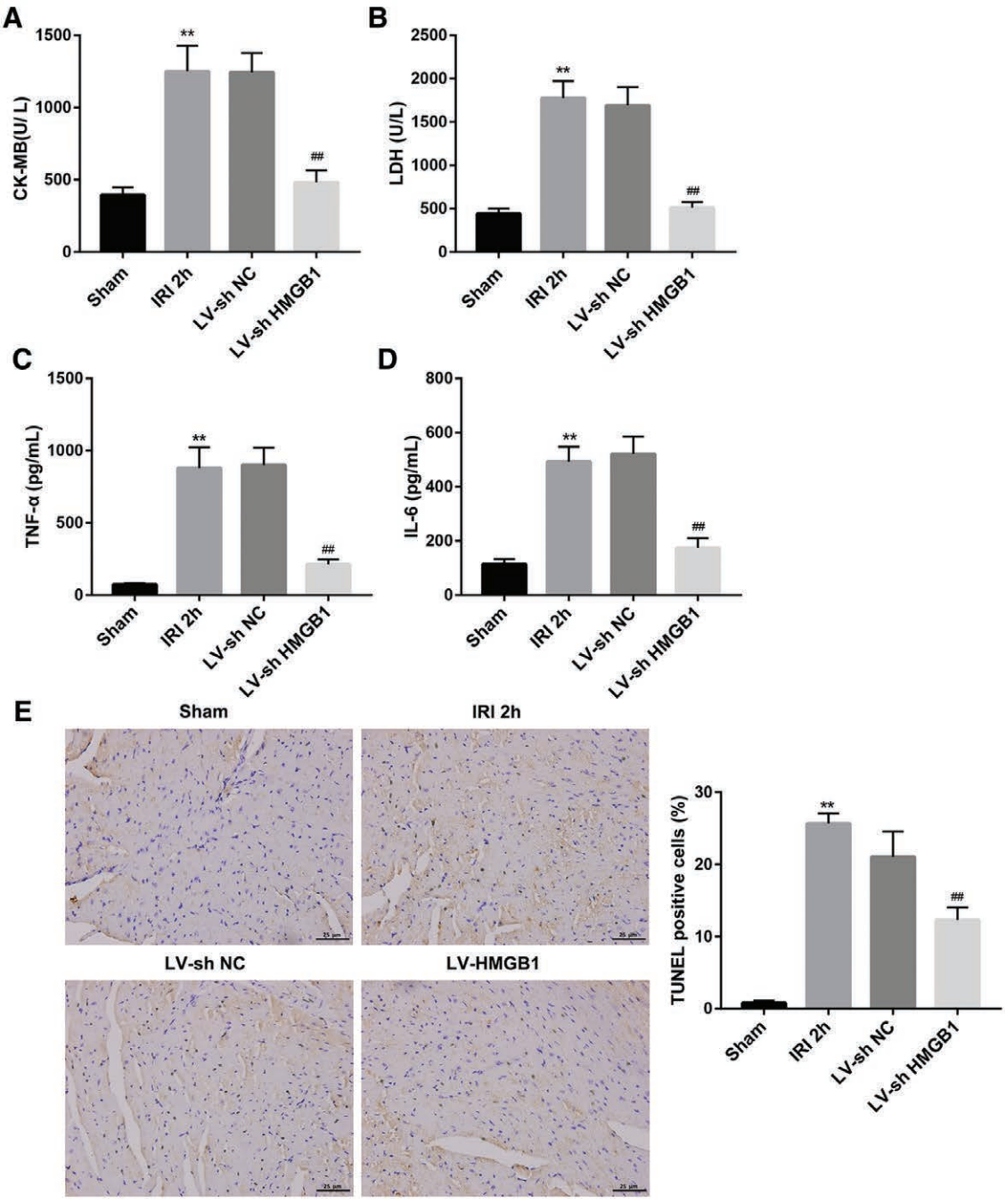

Fig. 4. Lentivirus-mediated shRNA interference targeting high-mobility group box 1 (LV-sh HMGB1) could exert protective effect on myocardial ischemia-reperfusion injury (IRI). Enzyme-linked immunosorbent assay (ELISA) detection of serum levels of creatine kinase muscle-brain fraction (CK-MB) (A), lactic acid dehydrogenase (LDH) (B), tumor necrosis factor-α (TNF-α) (C), and interleukin (IL)-1β (D) in Sprague-Dawley rats injected of LV-sh NC or LV-sh HMGB1 at 48 h before ischemia-reperfusion (I/R; reperfusion for 2 h) induction. E, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed to evaluate myocardial apoptosis. Scale bar: 25 μm. $^{**}p < 0.01$ vs the sham group; $^{\#\#}p < 0.01$ vs the LV-sh NC group. n = 6 for each group.

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